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13. ABSTRACT *(Maximum 200 Words)*

In this project, we attempt to establish the utility of an sntisense iron-responsive element (AS-IRE)-mediated gene expression system to targeting HER-2/neu-overexpressing breast cancer cells. During the first two years of funding, we have finished the proposed goal stated in **Task 1** by identifying the optimal HER-2/neu antisense IRE, i.e., AS-IRE4. Moreover, we showed preferential cell killing in HER-2/neu-overexpressing MDA-MB-453 cells using hTERT-AS-IRE4-Bax as opposed to in low HER-2/neu-expressing MDA-MB-468 cells (**Task 2**). The results obtained from **Task 1 and 2** have been published in ***Cancer Letters*** (174:151-158, 2001). To test the therapeutic efficacy of the hTERT-AS-IRE4-luc in a pre-clinical gene therapy model (**Task 3**), our next goal is to use a binary adenoviral vector Bax gene expression system to test the preferential killing of HER-2-overexpressing breast cancer cells *in vitro* and *in vivo*. To demonstrate the feasibility of adenovirus-based gene therapy in our orthotopic breast cancer xenograft model, we showed that adenoviral vector-mediated therapeutic gene transfer could yield treatment efficacy as indicated by our recent publication in ***Clinical Cancer Research*** (8:3290-3297, 2002). In addition, we have started to explore the possibility of using small interference RNA (SiRNA) as a novel approach to specific target HER-2-overexpressing cells. Our preliminary results suggested that SiRNA is potentially a powerful technology to achieve specific downregulation of HER-2/neu gene.

14. SUBJECT TERMS
Breast cancer, HER-2/neu overexpression, iron-responsive element, antisense, pre-clinical gene therapy

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INTRODUCTION

The overexpression of HER-2/neu proto-oncogene has been found in a variety of human cancers. In particular, amplification and overexpression of the HER-2/neu gene were found in 20-30% of patients with breast cancer. These patients had decreased survival and increased relapse rates. Therefore, HER-2/neu overexpression has been used as a poor prognostic indicator for patients suffering from this disease. To develop an expression system that targets the breast cancer cells overexpressing HER-2/neu mRNA, a novel approach is described that combines the antisense principle and the biochemical property of a translation regulator, an iron-responsive element (IRE). IRE, when placed 5 to a gene, functions as a negative translation regulator in that IRE interacts with iron-regulatory proteins (IRPs) and this protein-RNA complex blocks translation (1). One way to alleviate this translation inhibition is to prevent the IRE/IRP interaction by disrupting the IRE stem-loop structure via a sense-antisense hybrid. Thus, a HER-2/neu antisense IRE (AS-IRE) possessing the IRE consensus sequence and functioning as a translation inhibitor was generated. When placed 5 to a reporter gene, AS-IRE could direct the reporter gene expression in breast cancer cells that overexpress HER-2/neu mRNA, since the IRE-mediated translation inhibition can be overcome by the overexpression of HER-2/neu mRNA. In this project, we attempt to establish the utility of this novel approach to targeting HER-2/neu-overexpressing breast cancer cells. Our goals are: (1) to obtain an optimal AS-IRE that directs the maximum expression of the reporter gene in HER-2/neu overexpressing breast cancer cells; (2) to demonstrate a preferential killing of HER-2/neu overexpressing breast cancer cells by using the optimal HER-2/neu antisense IRE to direct the expression of a toxin gene encoding diphtheria toxin A-chain (DT-A); and (3) to test the therapeutic effect of the AS-IRE-mediated DT-A expression vector *in vivo* by treating the mice that bear tumors with or without the overexpression of HER-2/neu gene.

BODY

(Figures are attached in the Appendices)

Task 1: To obtain an optimal HER-2/neu antisense IRE.

We identified AS-IRE4 as the "optimal" AS-IRE in that AS-IRE4 behaves as a canonical IRE by interacting with IRP-1 and regulated by iron. Importantly, AS-IRE4 is able to direct a preferential gene expression in HER-2/neu-overexpressing breast cancer cells (2).

Task 2: To obtain an optimal HER-2/neu antisense IRE-regulated toxin gene (DT-A).

Using Bax (a pro-apoptotic protein in the Bcl-2 protein family (3)), as the therapeutic gene, directed by AS-IRE4 and a tumor-specific promoter of hTERT gene, we showed that hTERT-AS-IRE4-Bax could be used to achieve a preferential cell killing in breast cancer cells that overexpress HER-2/neu mRNA (2).

Task 3: To demonstrate the therapeutic effect of the HER-2/neu antisense IRE-mediated gene expression.

With a successful demonstration of preferential killing in HER-2/neu-overexpressing cells shown above (see **Task 2**), we will test the therapeutic efficacy of hTERT-AS-IRE4-Bax gene therapy treatment in an orthotopic breast cancer xenograft model. To achieve tumor-specific killing, we combined AS-IRE strategy with a human telomerase reverse transcriptase (hTERT) gene promoter (that is highly active in tumor cells but is repressed in normal primary cells) to direct the gene expression of a pro-apoptotic gene, Bax. We will use a binary adenoviral vector Bax gene expression system to test the preferential killing of HER-2-overexpressing breast cancer cells *in vitro* and *in vivo*. This viral gene expression system (4), developed by our collaborator, Dr. Bingliang Fang, Department of Thoracic and Cardiovascular Surgery, MDACC, consists of two adenoviruses (Ad): first, Ad-GT-Bax, it carries Bax gene controlled by a synthetic GAL4-responsive promoter (GT) that contains five GAL4-binding sites and a TATA box. GT is silent in 293 packaging cells and thus avoiding the toxic effects of Bax expression during virus production. Second, Ad-hTERT-GV16, it carries GAL4/VP16 fusion protein (GV16) that activates GT-Bax in tumors (but not in normal tissues) resulting in Bax expression and cell killing. We tested the binary adenovirus system and showed the overexpression of Bax in MDA-MB-468 breast cancer cells infected with Ad-GT-Bax and Ad-hTERT-GV16 as compared with the endogenous level of Bax expressed in cells infected with the control virus, Ad-Luc (Fig. 1). We will construct Ad-hTERT-GV16 and Ad-GT-AS-IRE-Bax (AS-IRE-Bax under the control of GT promoter). If our hypothesis is correct, we expect to see preferential killing of the HER-2-overexpressing breast cancer cells infected with Ad-hTERT-GV16 and Ad-GT-AS-IRE-Bax (Fig. 2).

To demonstrate the feasibility of using adenoviral vector-mediated gene transfer system in pre-clinical gene therapy for breast cancer, we constructed a recombinant adenoviral vector carrying a therapeutic gene, p202 (Ad-p202) (5). We showed that Ad-p202 treatment via intra-tumor (Fig. 3A) or systemic intra-venous (Fig. 3B) injection resulted in significant anti-tumor effect on an orthotopic breast cancer xenograft model derived from human breast cancer cell line MDA-MB-468. These results suggest that the use of adenovirus-based gene therapy is feasible in our experimental gene therapy system.

During the past year, we also explored other novel approach to target HER-2-overexpressing breast cancer cells, e.g., by downregulating HER-2 using RNA interference (RNAi) (6, 7). To test this idea, we generated small interference RNAs (SiRNA) that are specifically targeting HER-2 mRNA (Si-HER2) or the control luciferase mRNA (Si-GL3) (Fig. 4A). To test the specificity of S-RNA-mediated gene silencing, we performed a reporter assay in which a luciferase reporter gene driven by a TERT promoter (TERT-GL3-luc) was co-transfected with either Si-GL3 or Si-HER2 into 293 cells. As shown in Fig. 4B, we showed that Si-GL3 transfection completely abolished luciferase gene expression but not Si-HER2 or the TERT-GL3-luc alone (buffer). This observation confirmed the specific gene silencing effect by SiRNAs. To demonstrate the HER-2 specific gene silencing by Si-HER2, we co-transfected a HER-2 expression vector driven by a CMV promoter (CMV-HER2) with either Si-HER2 or the control Si-GL3 into 293 cells. Forty-eight hours post-transfection, cell lysates were isolated and analyzed for HER-2 protein expression by western blot. We showed that HER-2 protein expression is specifically downregulated in Si-HER2 transfected cells but not in those transfected with Si-GL3 or HER-2 expression vector alone (buffer) (Fig. 4C). These results clearly show th feasibility of using SiRNA-based treatment to target HER-2-overexpressing cells.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of AS-IRE4 as the "optimal" AS-IRE.
- Construction of hTERT-AS-IRE4-luc.
- Demonstration of iron-regulated gene expression using hTERT-AS-IRE4-luc.
- Demonstration of HER-2/neu overexpression-specific expression using hTERT-AS-IRE4-luc.
- Demonstration of HER-2/neu overexpression-specific cell killing using hTERT-AS-IRE4-Bax.
- Demonstration of the feasibility of using adenovirus-based gene transfer system to achieve treatment efficacy in breast cancer xenograft model.
- Demonstration of the feasibility of using SiRNA technology to specifically target HER-2-overexpressing cells.

REPORTABLE OUTCOMES

- Li, Z., Xia, W., Fang, B., and Yan, D.-H. (2001) Targeted gene expression in HER-2/neu-overexpressing breast and ovarian cancer cells by an antisense iron responsive element. Cancer Letters 174:151-158
- Ding, Y., Wen, Y., Spohn, B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Zheng, L., Hortobagyi, G. N., Hung, M.-C., and Yan, D.-H. (2002) Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer. *Clin. Cancer Res.* 8:3290-3297.

CONCLUSIONS

We have finished the proposed goal stated in **Task 1** and **Task 2**. The results have been published in ***Cancer Letters*** (174:151-158, 2001). To test the therapeutic efficacy of the hTERT-AS-IRE4-luc in a pre-clinical gene therapy model (**Task 3**), our next goal is to use a binary adenoviral vector Bax gene expression system to test the preferential killing of HER-2-overexpressing breast cancer cells *in vitro* and *in vivo*. We showed that adenoviral vector-mediated gene transfer system could yield therapeutic efficacy in our breast cancer orthotopic xenograft model as indicated by our recent publication in ***Clinical Cancer Research*** (8:3290-3297, 2002). In addition, we demonstrated the feasibility of using SiRNA as a novel approach to specific target HER-2-overexpressing cells.

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APPENDICES

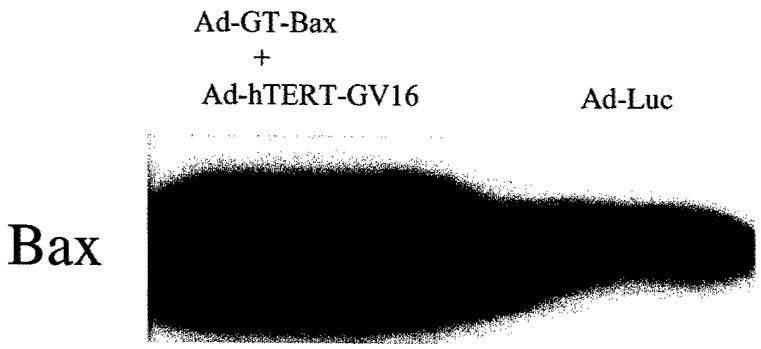

Fig. 1. The expression of Bax by co-infection with Ad-GT-Bax and Ad-hTERT-GV16. MDA-MB-468 breast cancer cells were infected with Ad-GT-Bax and Ad-hTERT-GV16; or the control virus, Ad-Luc. Forty-eight hours post-infection, the protein lysates were isolated for western blot analysis using anti-Bax antibody.

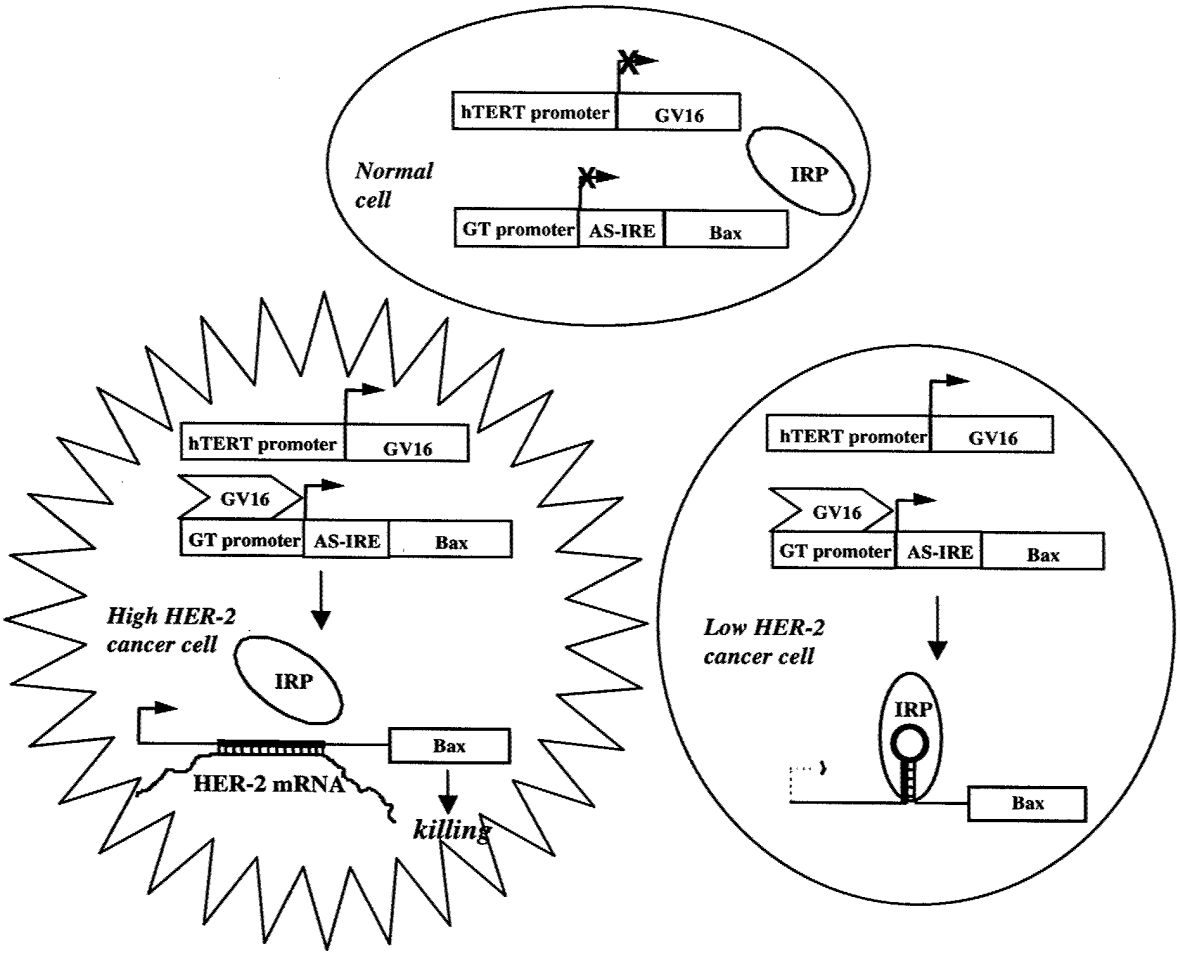

Fig. 2. Specific targeting the HER-2-overexpressing cancer cell by Ad-GT-AS-IRE-Bax/Ad-hTERT-GV16 binary adenoviral vector system. Co-infecting Ad-GT-AS-IRE-Bax and Ad-hTERT-GV16 into normal cell yields no cell killing due to the lack of GV16 expression because the hTERT promoter is inactive. In contrast, hTERT promoter is active in cancer cell and thus allows GV16 expression to activate GT promoter to transcribe AS-IRE4-Bax mRNA. However, AS-IRE4-Bax mRNA is not translated due to AS-IRE/IRP interaction in low HER-2 cancer cell. Such translational inhibition is alleviated in High HER-2 cell since HER-2 mRNA forms sense/antisense hybrid with AS-IRE4, and thus allows Bax expression leading to cell killing.

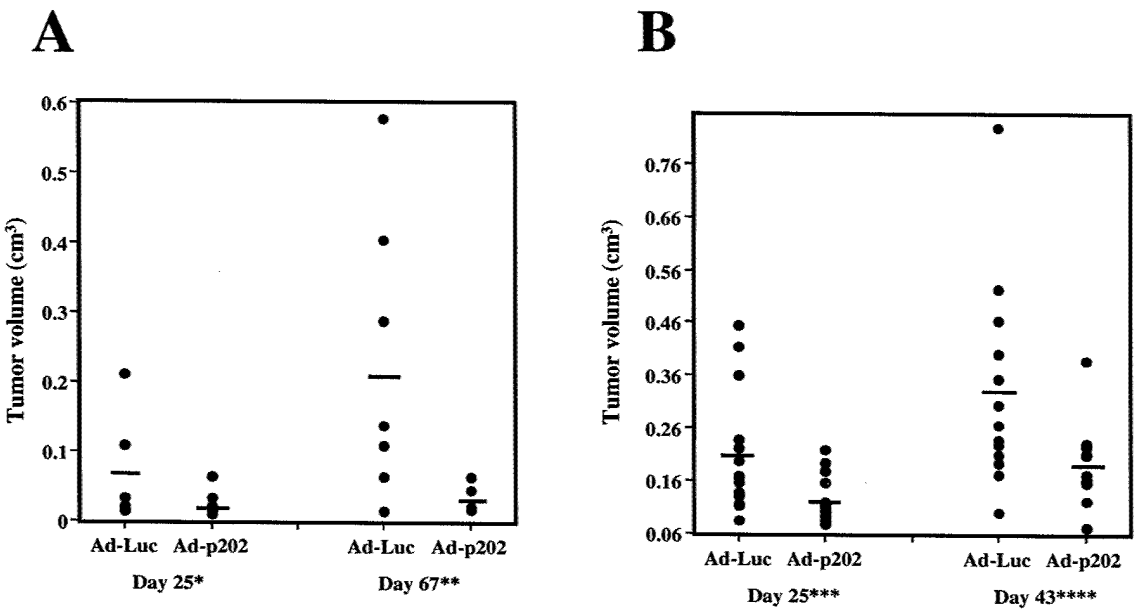

Fig. 3. Anti-tumor effect by systemic delivery of Ad-p202 on an orthotopic breast cancer xenograft model. *A*. Ad-p202-mediated anti-tumor effect on breast cancer xenografts by intra-tumor treatment. MDA-MB-468 cells (2 x 10^6 cells) were implanted in mammary fat pads of each female nude mouse. Tumor-bearing mice were divided into two treatment groups: Ad-Luc (total 7 tumors) and Ad-p202 (total 7 tumors), at 1 x 10^9 pfu per treatment via intra-tumor injection. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for seven weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, i.e., day 25 and day 67. *t*-test: * $p = 0.13$ and ** $p = 0.04$. *B*. Ad-p202-mediated anti-tumor effect on breast cancer xenografts by systemic treatment. MDA-MB-468 cells (2 x 10^6 cells) were implanted in mammary fat pads (2 tumors per mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups: Ad-Luc (Luc) (total 14 tumors) and Ad-p202 (p202) (total 14 tumors), at 5 x 10^8 pfu via tail vein injection. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for five weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, i.e., day 25 and day 43. *t*-test: *** $p = 0.0097$ and **** $p = 0.014$. *C*. Apoptosis correlates with p202 expression in Ad-p202-treated breast tumors. Mice were sacrificed 24 h after the last systemic treatment as described above. Tumors were then excised and fixed for the subsequent immunohistochemical analysis. p202 expression was analyzed by using antibody specific for p202 on tumor samples obtained from Ad-p202 or Ad-Luc treated mice (14). TUNEL assay was also performed to detect apoptotic cells in these tumors (15). The arrows indicate the representatives of apoptotic cells.

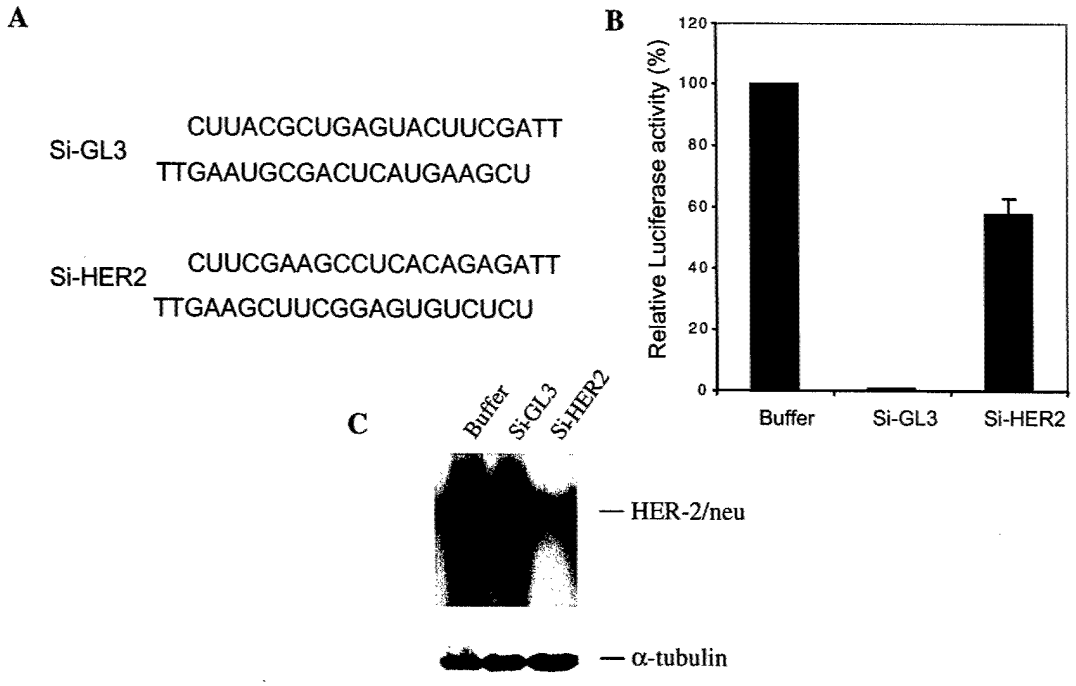

Fig. 4. HER-2 gene silencing by specific SiRNA. A. The sense (top) and antisense (bottom) sequences of the SiRNA duplexes specifically targeting GL3 luciferase (Si-GL3) and HER-2 (Si-HER2) mRNAs used in the experiment are shown. B. Specific luciferase gene silencing by Si-GL3. TERT-GL3-luc (2 μg), pRL-TK (0.1 μg) plasmid (Promega) and Si-HER2 or Si-GL3 (0.25 μg) were co-transfected into 293 cells. The luciferase activity was measured 20-h post-transfection using the Dual luciferase reporter assay kit (Promega). C. Specific HER-2 downregulation by Si-HER2. CMV-HER2 (3 μg) and Si-HER2 or Si-GL3 (0.25 μg) were co-transfected into 293 cells. The cell lysates were prepared 20-h post-transfection. Western blot analysis was performed using antibody against HER-2. The same membrane was then stripped and re-probed with α—tubulin antibody to serve as loading control.